

## Highly variable *Vitis* microsatellite loci for the identification of Pinot Noir clones

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### Summary

Nineteen new microsatellite loci of *Vitis* were elaborated by following the procedure of tagging an SSR-enriched library. Primers for these VRG markers were used for genotyping grapevines. Only the markers VRG 1, VRG 2, VRG 4, VRG 7, VRG 9, VRG 10, VRG 15 and VRG 16 show heterozygous alleles and Mendelian segregation. Other VRG loci such as VRG 5, VRG 6, VRG 11, VRG 12, VRG 13 and VRG 17 produce a multiallelic profile and some of them show distorted segregation. Variability of the VRG loci is rather high as compared to other grapevine SSR markers. Stable VRG markers such as VRG 16 can be useful for the identification of cultivars. Highly variable VRG microsatellites could be successfully applied to trace polymorphism within the variety Pinot Noir. Clones of Pinot Noir could be differentiated using these markers. By applying the PhyQuest program, a dendrogram showing the genetic divergence within Pinot Noir clones was constructed.

**Key words:** *Vitis*, clonal selection, genotyping, Pinot Noir.

### Introduction

Pinot Noir is one of the major traditional grapevine cultivars worldwide with a long historical background. About 40,000 ha are cultivated mainly in temperate zones. Due to the large family and several closely related cultivars, the genetic relationship among the Pinots was not clear (AMBROSI *et al.* 1994). Application of SSR (Simple Sequence Repeats) markers revealed the relationship of the Pinots and helped to clarify their origin (REGNER *et al.* 2000 a). The high variability within cv. Pinot Noir has also generated other perspectives in regard to the genetic relationship of Pinot-related cultivars (FORNECK *et al.* 2002). It was supposed that Pinot Meunier is not an individual cultivar but a chimeric vine (FRANKS *et al.* 2002). Chimeric types of Pinot Gris are supposed to be the origin of all Pinots (HOCQUINGY *et al.* 2004). Even Pinot Noir, Pinot Gris and Pinot Blanc belong to the same cultivar, their main difference being berry color.

Microsatellite markers became widely accepted for genotyping grapevines with the aim to identify cultivars (THOMAS *et al.* 1993, TESSIER *et al.* 1999, SEFC *et al.* 2001). Due to their high degree of polymorphism, their codomi-

nant inheritance and their reproducibility, SSR markers are preferred to other DNA based markers (VOSMAN *et al.* 2001). In fact, internationally accepted scoring systems based on these markers are already introduced to improve germplasm management (THIS *et al.* 2004).

Most stable SSR markers are located in noncoding areas due to their two nucleotide repeats. SSR markers, which are derived from EST (Expressed Sequence Tag) or cDNA databases, could be aligned with higher relevance to the phenotype of the vine. The sequence of most SSRs seems to be highly conserved as usual in noncoding areas. Therefore utility of SSRs is primarily given for genotyping cultivars of grapevine and not for detecting spots (BORREGO *et al.* 2002).

Since grapevines are cultivated since ancient times, several traditional varieties like Pinot show a high degree of variability (BASSERMANN-JORDAN 1975). The level of variability depends on the cycles of propagation, the spread to different locations and the intensity of cultivation by growers (SCHÖFFLING and STELLMACH 1993). Since the 19<sup>th</sup> century clonal selection has been used to improve agricultural performance of several grapevine cultivars (AMBROSI *et al.* 1994). In clonal selection the natural variation of a grapevine cultivar is used for specific viticultural tasks enabling breeders to offer different phenotypes within the term of a single cultivar. For this reason, clones nowadays do not represent a huge spectrum of variability within a traditional variety but reflect the result of selection criteria by viticulturists. For breeding purpose it would be advantageous to identify propagation material of individual clones and it would also be helpful to estimate the heterozygosity of the involved material and to compare new genotypes with clones already established. A further interest is to gain an indication of genetic differences of these clones and to demonstrate the variability of individual Pinot Noir clones.

The first results from differentiating clones by genetic markers (BELLIN *et al.* 2001, REGNER *et al.* 2001, RIAZ *et al.* 2002) show that this topic is not limited by tools but by the amount of loci applied. If many markers are used the probability to find differing alleles increases. RAPD (Random Amplified Polymorphic DNA), Inter SSR, AFLP (Amplified Fragment Length Polymorphism) and even SSR markers could be applied to find polymorphism in White Riesling (REGNER *et al.* 2000 b), Traminer (IMAZIO *et al.* 2002; REGNER *et al.* 2002) and other cultivars (HOCQUINGY *et al.* 2004). For identification of clones only sequence-characterized markers will be reproducible and stable in analysis.

## Material and Methods

Construction of a library and elucidation of the SSRs. Genomic DNA of *Vitis riparia* was purified from leaves of *in vitro*-grown plants. Isolation was performed following the procedure published by THOMAS *et al.* (1993). DNA restriction fragments with sizes of 200-400 bp were introduced into the multicloning site of a pUC 18 vector. Positively transformed clones of the *E. coli* XL1 blue strain were screened first for insertions by testing  $\beta$ -galactosidase production and afterwards were tested by hybridisation against a (GA)<sub>n</sub> probe. The sequences were developed in the frame of the VMC (Vitis Microsatellite Consortium = Collaboration Agreement for the Development of Grape Microsatellite Markers 1998).

Segregation analysis and genotyping. For the segregation analysis of the developed VRG (Vitis Riparia Götzhof) markers, 68 offsprings derived from a cross of Welschriesling  $\times$  Sirius were used. They are all kept at the Department for Grapevine Breeding at the Federal College and Institute for Viticulture and Horticulture in Klosterneuburg (HBLA u. BA Klosterneuburg). In order to prove their utility in genotyping, these markers were also analyzed in 45 traditional cultivars well established in the HBLA u. BA Klosterneuburg (Tab. 1).

DNA was extracted from young leaves of field-grown plants following the protocol of THOMAS *et al.* (1993) modified by REGNER *et al.* (1998). Amplification was performed in 20  $\mu$ l of the buffer solution, which consisted of 16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 67 mM Tris-HCl, pH 8.8, 1.5 mM MgCl<sub>2</sub>, 0.01 % Tween 20, 0.1 mM of each dNTP (GenXpress, Maria Wörth, Austria), 0.2  $\mu$ M primer (labelled with the fluorescent dyes 6FAM, TET and HEX), 1 Unit Biotherm Taq DNA polymerase (GenXpress, Maria Wörth, Austria), and 50 ng of genomic DNA of grapevine. Labelling with different fluorescent coloring agents facilitated the application in multiplex PCR assays. The amplification of the SSR loci was performed in an Omnigene (Hybaid, Teddington, Great Britain) thermocycler processed for 36 cycles. The general PCR protocol applied was 2 min denaturation at 94 °C and 35 cycles with an annealing phase of 30 s and temperatures between 45 °C and 55 °C, 1 min synthesis phase at 72 °C and denaturation for 15 s at 92 °C. The annealing temperature for each locus was set according to the T<sub>m</sub> of forward and reverse primer -10 °C temperature. A final extension of the fragments was performed at 72 °C for 5 min.

Yield of DNA fragments was estimated by running an aliquot of the sample on a 2 % agarose gel stained with ethidium bromide. The samples were denaturated by heating up with formamide and loaded together with a size standard (Genescan 350 Tamra, Appl. Biosystems, Warrington, Great Britain) on 6 % polyacrylamid gel. Detection of the SSR fragments was carried out by an automated sequencer (ABI 373, Perkin-Elmer, Vienna). Analyses were done twice in the case of polymorphism with a different plant sample. Characterization of Pinot Noir clones. Thirteen Pinot Noir clones were used in this study. They are all kept at the HBLA u. BA in Klosterneuburg. Their specific

Table 1

Fortyfive traditional grapevine cultivars used for the characterisation of VRG SSR loci

Silvaner	Portugieser Blau
Chasselas	Muskat Ottonel
Traminer	Perle von Csaba
St. Laurent	Sauvignon Blanc
Cabernet Franc	Semillon
Veltliner Frührot	Rotgipfler
Chardonnay	Burgunder
Neuburger	Roesler
Wildbacher Blau	Rathay
Blauburger	Jubiläumsrebe
Bianca	Goldburger
Müller-Thurgau	Muskateller
Grenache	Heunisch
Schwarzriesling	Aligote
Steinschiller	Orangetraube
Merlot	Veltliner Grün
Lambrusco	Cabernet Sauvignon
Veltliner Rot	Zierfandler
Blaufränkisch	Riesling
Bouvier	Sultanina
Königin der Weingärten	Sangiovese
Scheibkern	Seifert
Vernatsch	

Table 2

Pinot Noir clones used in this study. Selection criteria and specific origin

No.	Pinot Noir clones	Selection criteria	Origin
1	Fr 52-86	Typical Pinot	Freiburg (D)
2	Fr 52-57	Intensive colour	Freiburg (D)
3	Gm A	Low yield	Geisenheim (D)
4	Kl 5/43	High wine quality	Klosterneuburg (A)
5	Mariafeld	Loose cluster	Wädenswill (CH)
6	W2/10	High yield	Wädenswill (CH)
7	Kl 3/45	Wines of high typicity	Klosterneuburg (A)
8	Fr 52-80	Typical Pinot	Freiburg (D)
9	Re 16/9	Small bunches	Retz (A)
10	Poly VI	High yield	Unknown
11	Oberlin	Low yield	Pully (F)
12	Re 14/13	High wine quality	Retz (A)
13	W 10/5164	High yield	Wädenswill (CH)

origin and their selection criteria are shown in Tab. 2. The grapevines were evaluated according to the O.I.V descriptors (O.I.V. 1983) and were analyzed with 35 SSR markers commonly used in identification of grapevine cultivars. These included VVS markers developed by THOMAS and

SCOTT (1993), VVMD markers by BOWERS *et al.* (1996, 1999) and VRZAG markers by SEFC *et al.* (1999). With the aim to find polymorphism within the cultivar, all newly developed SSR (VRG) markers were also applied. The protocol for DNA extraction, amplification and detection of the SSR fragments was the same as described above.

**Statistical analysis:** The calculation of the SSR based heterozygosity index was performed by using the software PhyQuest (TIEFENBRUNNER *et al.* 2002). A distance matrix could be calculated to obtain a dendrogram where the genetic similarity was observed.

## Results and Discussion

Nineteen VRG SSR clones were sequenced and primers were designed for the amplification of these loci (Tab. 3). Their segregation pattern was analyzed in 68 seedlings derived from a cross of Welschriesling x Sirius as shown in Tab. 4. Mendelian segregation of heterozygous alleles could be detected in VRG 1, VRG 2, VRG 4, VRG 7, VRG 9, VRG 10, VRG 15 and VRG 16. VRG 15 shows distorted behavior in the small scale segregation analysis. The loci VRG 5, VRG 6, VRG 11, VRG 12, VRG 13 and

Table 3

Primer Sequences, sizes in base pairs (bp) and melting temperature (T<sub>m</sub>) for the amplification of 19 VRG SSR loci and their repeat type

Name (VMC code)	Sequence	Size (bp)	T <sub>m</sub> (°C)	repeats
VRG1F	AAggTTTccTgccggcgATAAcc	23	72	(TC)18x
VRG1R	ccATTggTAAATAAAgTccc	20	56	
VRG2F	cTccTccgcTcAcTcAccATTTTc	24	69	(TC)16x
VRG2R	cATgccTgcAggTcgAcTcTAgAgg	25	71	
VRG3F	ggTAcccggggATcTAcTAc	20	59	(GA)34x
VRG3R	AcAgcAAAAcgcAgccc	17	63	
VRG4F	gTTcATcTgTcTAAAccTAggg	22	56	(GA)17x
VRG4R	AcAAgcTgcTTAcTccgcTccgg	23	71	
VRG5F	gATcccTTcATgcTATcccATggc	24	70	(TC)19x
VRG5R	AgcTTgcATgccTgcAggTcgAcTc	25	75	
VRG6F(VMCNG3a7F)	AgcccggAATcATAgcTcAc	20	64	(GA)39x
VRG6R(VMCNG3a7R)	ggcTgTcgggTAAccTcc	18	63	
VRG7F(VMCNG3c8F)	gcgATgAcgTccAATccAc	19	65	(GA)35x
VRG7R(VMCNG3c8R)	cccATTcTATcgcTcccAgT	20	63	
VRG8F(VMCNG3f8F)	AAgggATgATggTgTgcAAc	20	63	(GA)46x
VRG8R(VMCNG3f8R)	cTcAgcTcTcTcTcTAGgcTcTTAc	25	61	
VRG9F(VMCNG3h8F)	AAgATAATgggAAggAgAAcActg	24	62	(TC)10+5x
VRG9R(VMCNG3h8R)	ATgATggATTccccTTAgAc	22	64	
VRG10F(VMCNG3a10F)	gTgccTTgTcTTcAccTTAgcc	22	64	(TC)26x
VRG10R(VMCNG3a10R)	TTTgTgAgAAgTggAggTcggA	22	64	
VRG11F(VMCNG3c10F)	cTgcAcTTgATgATcAcAAAgA	22	61	(GA)34+13x
VRG11R(VMCNG3c10R)	gTgAgATcgAcTTgcgATAgAg	22	61	
VRG12F(VMCNG3e10F)	TcAggAgTTAgccATcAgggTc	22	64	(GA)40x
VRG12R(VMCNG3e10R)	ggccTAcATAggcAgggAAgT	21	64	
VRG13F(VMCNG3g10F)	TTTgggTggATTccTTTTTA	20	59	(GA)35+3x
VRG13R(VMCNG3g10R)	TcTgcTTTcTcTcTgTcTTg	22	61	
VRG14F(VMCNG3a11F)	AccAgcggcAATAATgTcAgT	21	64	(TC)13x
VRG14R(VMCNG3a11R)	gAccTcgTggTcgAccgA	18	66	
VRG15F(VMCNG3b11F)	cAggccgAgTTTcAaccggT	19	66	(GA)34x
VRG15R(VMCNG3b11R)	AggATcccgcggc	14	66	
VRG16F(VMCNG3f11F)	AATTcTAcAAccATgAgTccc	22	61	(GA)18 x
VRG16R(VMCNG3f11R)	TTATcTcAccAAAgcTcTTccA	22	66	
VRG17F(VMCNG3a12.1F)	AATgcAAgTggcAAgcAATgAc	22	66	(TC)5+2x
VRG17R(VMCNG3a12.1R)	cTgcTgcTTTTcATgTcggTTT	22	66	
VRG18F(VMCNG3b12F)	ccTggTTcAcAATTTcggcT	20	64	(GA)46x
VRG18R(VMCNG3b12R)	TgTcAgTgAccgcAcTaccg	20	65	
VRG19F(VMCNG3f12F)	TTAgcccgTgAATAAgAAgTg	22	62	(TC)29x
VRG19R(VMCNG3f12R)	ggccTTAcTAGTAAccgTgTgc	22	62	

Table 4

Segregation pattern of VRG markers analyzed in 68 offspring derived from a cross Welschriesling × Sirius. Distribution of parental alleles and missing alleles in the seedlings was calculated ( $p > 0.05$ , degree of freedom = 1). H (primary allele). A (secondary allele)

SSR locus	Observed H	Observed A	Missing values	Total	Chi-square value of distribution
VRG 1	34	28	6	68	0.58
VRG 2	25	43	0	68	4.76
VRG 3	53	10	5	68	29.34
VRG 4	28	38	2	68	1.51
VRG 6	27	41	0	68	2.88
VRG 7	34	34	0	68	0.00
VRG 9	39	26	3	68	2.60
VRG 10	28	39	1	68	1.81
VRG 11	32	35	1	68	0.13
VRG 15	47	19	2	68	11.87
VRG 16	34	34	0	68	0.00

VRG 17 produce multiallelic profiles and VRG 5 and VRG 12 show distorted segregation. At VRG 3, VRG 13, VRG 14, VRG 19 no stable inheritance in the population was observed. Although VRG 3 and VRG 13 could be used for genotyping in Pinot, only few of the descendants bear the same alleles as their parents at these two loci. VRG 8 and VRG18 did not result in stable amplification of the micro-satellite DNA (Tab. 5).

Analysing these markers in 45 traditional cultivars (Tab. 1) their potential in genotyping grapevines could be estimated. VRG 9 was the most useful marker for the identification of grapevines due to its polymorphism, distribution of alleles (Tab. 5), incidence in all used grapevines and stable inheritance of the locus.

Even though morphological and agronomic differences were found and used as selection criteria for the 13 Pinot Noir clones studied, both O.I.V. descriptors (data not shown) and the analysis with VVS, VVMD and VRZAG markers (Tab. 6) confirmed the “trueness to type” of these clones. These published markers, often used in identification of grapevine cultivars, did not indicate any polymorphism. They show stable alleles within the Pinot clones and seem to be useless for detecting intravarietal genetic variation. They were selected out of a pool of markers due to their experimental stability.

Nevertheless, some of the developed VRG markers, resulted in different allelic profiles (Tab. 7). The polymorphism of differing alleles could be reproduced in a second

Table 5

Alleles of 8 VRG markers: Size in base pairs (AS), frequencies in traditional cultivars (Tab. 1) (AF), expected ( $H_e$ ) and observed ( $H_o$ ) heterozygosity in 45 grapevines

VGR 1		VGR 2		VGR 3		VGR 4		VGR 9		VGR 10		VGR 15		VGR 16	
AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF
198	0.1	108	0.08	196	0.03	107	0.24	224	0.2	91	0.02	136	0.4	246	0.5
221	0.02	155	0.2	197	0.3	150	0.5	225	0.25	93	0.2	140	0.08	250	0.02
224	0.4	157	0.08	198	0.1	193	0.2	226	0.01	95	0.3	142	0.08	254	0.02
226	0.18	158	0.02	212	0.02	195	0.06	232	0.02	97	0.03	148	0.02	260	0.1
228	0.2	159	0.4	214	0.1	198	0.02	236	0.06	101	0.1	150	0.2	261	0.1
230	0.1	162	0.2	240	0.44			237	0.02	103	0.06	164	0.12	263	0.26
	167	0.02					240	0.07	107	0.06	186	0.1			
							241	0.05	109	0.04					
							242	0.01							
							246	0.11							
							247	0.08							
							248	0.11							
$H_e$	0.74		0.74		0.69		0.64		0.85		0.84		0.73		0.66
$H_o$	0.68		0.75		0.65		0.51		0.83		0.77		0.52		0.58



Table 6

Allelic profiles (sizes in base pairs) at 35 stable SSR loci (VVS, VVMD, VRZAG) analyzed in 13 Pinot Noir clones. Due to the experience of mapping different populations we could determine VVMD6 as a homozygous locus while VRZAG 12 shows a null allele

Locus	Pinot Noir clones (1-13)	
VVS 1	183	190
VVS 2	137	151
VVS 3	214	220
VVS 4	168	173
VVS 29	171	179
VVMD 5	228	238
VVMD 6	199	199
VVMD 7	239	243
VVMD 8	140	142
VVMD17	212	220
VVMD 21	249	249
VVMD 24	216	218
VVMD 25	243	253
VVMD 26	251	257
VVMD 27	185	189
VVMD 28	221	239
VVMD 31	216	216
VVMD 32	241	273
VVMD 34	254	264
VVMD 36	254	254
VRZAG 7	157	157
VRZAG 12	153	
VRZAG 15	167	179
VRZAG 21	202	208
VRZAG 25	227	238
VRZAG 29	114	118
VRZAG 30	151	151
VRZAG 62	189	195
VRZAG 64	140	164
VRZAG 67	128	154
VRZAG 79	240	246
VRZAG 82	251	271
VRZAG 83	190	202
VRZAG 93	188	188
VRZAG 112	242	244

trial and the results were confirmed with a second sample of each clone. The genetic differences are not surprising, however, the possibility to find some deviations at SSRs is rare (HOCQUINGY *et al.* 2004). Mutations located in the range of an SSR marker characterized by the sequence enabled us to use them for clonal identification. Besides the VRG marker data also some other SSR polymorphism within the Pinot clones could be detected (data not shown). It seems that the SSR variability within a cultivar depends on the age and frequency of propagation of the cultivar as well as on the location in the genome. In mapping studies of Welschriesling x Sirius higher variable regions could

be illuminated. The occurrence of null alleles or the loss of heterozygosity as seen in clone Fr 52-57 at locus VRG 2 (Table 7) is one main source for polymorphic SSR loci. The appearance of a third or fourth allele due to chimerism is another kind of variability (HOCQUINGY *et al.* 2004). Mutations at the annealing site can easily inhibit the amplification of the allele and cause null alleles. A null allele was accepted if the repetition resulted in the same marker size without changing the PCR protocol for easier annealing conditions. Usually homozygous alleles would be recognized by higher amounts of fragment DNA. The formation of new alleles at VRG 2 could be observed in Fr 52-86 and Kl 5/43. At several other VRG loci new allele sizes can be observed due to changes in the DNA sequence. If the size is out of the frame of the locus, a larger rearrangement in the genome could have taken place. A third allele with the expected length for that locus could have its origin in periclinal chimera (FRANKS *et al.* 2002).

All these deviations make an identification system of clones within the variety feasible. Each genotype differs from all others at least by combining several SSR loci. As clonal material is derived by vegetative propagation these changes happen during cell division.

Polymorphism was used to calculate a proximity matrix of the clones. The clustering analysis based on these data (Figure) showed that clone Re 16/9 represents the most individual genotype, followed by W 2/10, Kl 3/45, Fr 52-80. Re 16/9 was collected from an ancient vineyard with various cultivars near Retz (Lower Austria). Clones from Freiburg were situated closely together in the dendrogram, maybe due to their similar selection area. As the groups contain either local individuals, loose cluster types or dark color types, the morphological range of Pinot Noir could be confirmed as a very broad one. Nowadays it can be supposed that the same clonal individuals have been dispersed all over Europe.

Genetic analysis as a tool to verify differences between clones is a useful method for breeding. Most grape growers prefer to cultivate clonal material of a traditional cultivar. In France and other countries with controlled production systems (appellation d'origine contrôlée) wines must be produced from specific clones to be accepted for the common A.O.C. labelling. Therefore identification of clonal material is highly appreciated for clonal breeding. While RAPD, AFLP, InterSSR and MSAP (Methylation-Sensitive Amplified Polymorphism) (IMAZIO *et al.* 2002) markers produce higher levels of polymorphism, SSR markers are more appreciated due to their stability, reproducibility and known position in the genome (ADAM-BLONDON *et al.* 2004). For clonal identification it will be very helpful to gain SSR markers like the VRGs, which are tagging higher variable regions in the genome.

The chance to identify an individual clone raises the question of any clonal protection. Comparing the costs of transgenic plants and traditionally bred clones, it is not logical that genetically modified (GVO) grapevines should be patented whereas clones do not get a proper protection. It would be very helpful if private growers would offer their clonal material to others. But they will not do so without the prospect of any benefit. Therefore interesting genetic

Table 7

Allele sizes (base pairs) at the VRG SSR loci analyzed in 13 Pinot Noir clones. Boldface numbers are deviating from the main profile of Pinot Noir. Unique alleles are favored for the identification of a specific clone. Corresponding clone numbers are listed in Tab. 2

Clone	VRG 1	VRG 2	VRG 3	VRG 4	VRG 7	VRG 9	VRG 10	VRG 11	VRG 13	VRG 15	VRG 16										
1	221	226	155	<b>158</b>	240	198	164	194	226	248	119	110	128	151	<b>199</b>	248	262				
2	<b>198</b>	<b>226</b>	155	240	198	164	194	226	248	119	94	105	<b>114</b>	128	<b>174</b>	<b>95</b>	<b>139</b>	151	248	262	
3	221	226	155	162	240	<b>297</b>	<b>339</b>	164	194	226	248	119	<b>110</b>	128	<b>95</b>	<b>139</b>	151	<b>199</b>	248	262	
4	221	226	155	<b>158</b>	198	194	194	226	248	119	94	<b>114</b>	128	<b>95</b>	151	<b>95</b>	151	<b>199</b>	248	262	
5	221	226	155	162	198	164	194	226	248	119	105	105	128	<b>95</b>	<b>139</b>	151	<b>199</b>	248	262		
6	221	226	155	162	<b>197</b>	<b>214</b>	240	<b>150</b>	194	226	248	119	99	105	<b>95</b>	<b>139</b>	151	<b>199</b>	248	262	
7	221	226	155	162	240	198	<b>164</b>	194	226	248	119	99	105	128	<b>95</b>	<b>139</b>	151	<b>199</b>	248	262	
8	221	226	155	162	198	194	194	226	248	119	94	<b>110</b>	114	128	<b>95</b>	<b>139</b>	151	<b>199</b>	248	262	
9	221	226	155	162	<b>107</b>	<b>198</b>	<b>164</b>	194	<b>268</b>	<b>274</b>	119	94	<b>110</b>	114	128	<b>95</b>	<b>139</b>	151	<b>199</b>	248	262
10	<b>198</b>	<b>224</b>	155	162	198	<b>164</b>	194	226	248	119	105	128	128	151	151	151	151	151	151	248	262
11	221	226	155	162	240	198	194	226	248	119	105	<b>114</b>	128	151	151	151	151	151	151	248	262
12	221	226	155	162	198	194	194	226	248	119	<b>131</b>	94	105	<b>100</b>	<b>114</b>	128	151	151	151	248	262
13	221	226	155	162	240	198	194	226	248	119	<b>131</b>	105	<b>110</b>	128	151	151	151	151	151	248	262

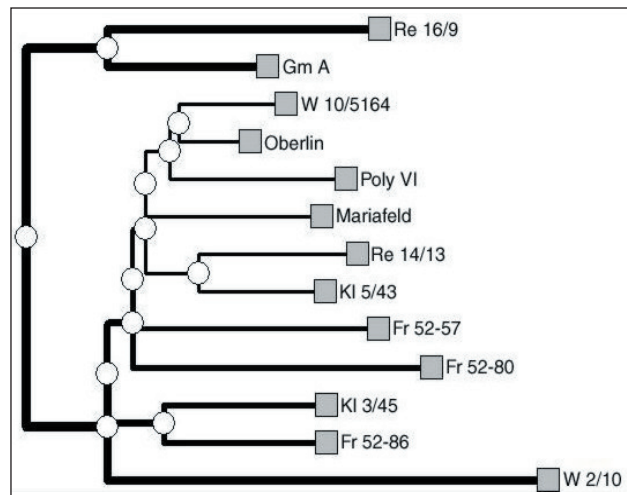


Figure: Dendrogram generated using the proximity matrix from the VRG SSR data of 13 Pinot Noir clones. The distance was calculated following Genelocus with hierarchical weighing of states.

individual clone could be the use of plants with defined unique sequences. Usually transgenic plants are accepted for patenting due to their additional transferred sequence. If a clone can be differentiated from all the others by determining a specific sequence there is no reason to refuse this protection approach.

Hopefully, concerning the proceedings in genetic mapping and definition of specific clones, we will reach the point that mutations can easily be linked to the corresponding DNA. The DUS (distinctness, uniformity, stability) test as criterion for variety protection will be applicable even to clonal material by using highly variable SSR markers. Moreover this genetic information about a clone allows to avoid duplicates in clonal collections and enables control of clonal identity.

What are the possible sources of the variation and their spread in grapevine? Repetitive sequences like SSRs are more frequently mutated than others. The slippage of one or more additional repeats is supposed (RAFALSKI *et al.* 1996). Even the enlargement of SSR loci during evolution has been postulated (ORTI *et al.* 1997). Nevertheless, the flanking regions of the SSR insert could also be changed. The annealing process of the primers at the conserved sites depends on the coincidence of the sequence. Mismatches could avoid amplification. If the mutated alleles and the original ones are coexisting in a vine, more than two alleles would be observed. It is supposed that in this case a chimeric grapevine would be detected. During longer propagation periods chimeric vines also may be separated into two different genotypes. This would explain the finding of different allelic profiles within samples of a single variety. The only risk for this procedure is that new mutations occur due to *in vitro* manipulations or spontaneous events.

The variability of Pinots (Gris, Blanc, Noir and Meunier) led to the assumption that Pinot Gris is the origin of the Pinots (HOCQUINGY *et al.* 2004). Due to the limited use of SSR loci and the non-representative sample for the whole development of the Pinots, it can be supposed that

material is kept away from the wine community due to the lack of any financial benefit for clonal selection.

Hence, if the genetic analysis reveals that the genetic basis of an individual clone differs from all other registered clones, the prerequisites of novelty are fulfilled. Additionally, for any kind of protection, uniformity and stability must be shown. In the past these criteria could not be evaluated for clones. Despite morphological differences, differentiation of clones is neglected by the International Union for Protection of Varieties (UPOV). Nowadays UPOV does not accept genetic differences as the only criterion for showing distinctness. One possible way to protect an

research on the variability of Pinots should be intensified. There is a lack of connecting the genetic profile with the corresponding clones; therefore no comparison is possible. Future work on variability within a cultivar is requested to present data as a guideline for identification. Clonal profiles shown in this report are easily reproducible.

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